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AN OSMIC IMPREGNATION METHOD FOR MITOCHONDRIA IN PLANT CELLS

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Osmium tetroxide, in technics like Kolatchev's (1927–8) and its various modifications, has been used for years in animal cytology for the preservation of various protoplasmic constituents, such as the Golgi apparatus. Bowen (1927–8), using the Kolatchev technic on plant tissues, described a new category of cytoplasmic inclusions

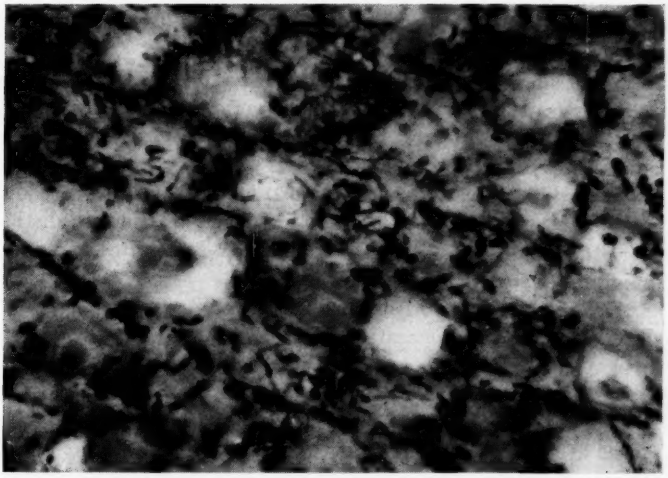

Fig. 1. Photomicrograph of root-tip cells of *Pisum*, × 1425. The preparation is unstained.

which he called the "osmiophilic platelets." The author (1940), in duplicating some of Bowen's work, used this technic and observed the bodies described by Bowen which he considered to be deformed chondriomes caused largely by the initial fixation. A simple modification of the technic, however, in which Zirkle's fluid was substituted for Champy's, resulted in preparations in which the chondriome and

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proplastids were beautifully preserved and selectively blackened by the osmium tetroxide, showing clearly the transition stages between the two categories. No staining is necessary after bleaching, the chondriome and proplastids appearing jet black against the light gray background of cytoplasm and nuclei (Fig. 1). The nuclei, cytoplasm and cell walls can be stained with Altmann's mixture of acid fuchsin and anilin oil (Lee, 1937, p. 305) followed by a counterstain of gold orange in clove oil. The result is a brilliant triple stain, with the nuclei red, cytoplasm and cell walls orange, and the chondriome black.

A summary of the technic is as follows:

1. Cut root-tips or other material directly into Zirkle's[2] fixative and fix for 48 hours.

2. Wash 8 hours or over night in running tap water.

3. Treat with 2% OsO_4 for 4–6 days—change solution on alternate days. (It was found impossible to standardize this step of the technic, because root-tips in the same bottle will exhibit different rates of reduction of the OsO_4.)

4. Wash 8 hours or over night in tap or distilled water.

5. Dehydrate by the usual alcoholic series substituting benzene for xylene in clearing, or by the shorter *N*-butyl-alcohol–ethyl-alcohol series of Lang (1937).

6. Embed in 52° paraffin and section at 5 μ.

7. Bleach as follows: 1% $KMnO_4$, 5 minutes. Rinse in distilled water, then place in 3% oxalic acid for 2–3 minutes. Wash in running tap water for 15 minutes.

8. Dehydrate thru graded alcohols, clear in xylol and mount in balsam. Or, if the preparation is to be stained, pass from water into acid fuchsin (pipette acid fuchsin on slide, heat until steaming, let stand for 5 minutes, repeating the procedure once more, and when cool, wash in water), and ascend thru the alcohols, counterstaining in 1% gold orange in clove oil after absolute alcohol. After the counterstain, wash and clear in xylol and mount in balsam.

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[2]Zirkle's fixative:

3% $K_2Cr_2O_7$	1.25 g.
3% $(NH_4)_2Cr_2O_7$	1.25 g.
3% $CuSO_4$	1.00 g.
Distilled water	100.00 g.

THE DETERMINATION OF APPARENT ISOELECTRIC POINTS OF CELL STRUCTURES BY STAINING AT CONTROLLED REACTIONS[1]

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ABSTRACT.—**The staining reactions at controlled pH-values of various dyes with the nucleus and cytoplasm of *Trichonympha collaris* under different conditions were investigated. When staining intensity was plotted against pH, it was found that with each dye a different curve was obtained. "Isoelectric points" obtained by superposition of acid and basic dye curves varied for the same material with the dyes employed. It was found that, with the same dye, the curves of staining intensity plotted against pH varied with the buffer system utilized. Moreover, the intensity of staining at any pH was found to vary directly with the concentration of dye and inversely with the concentration of buffer. Various factors modifying staining intensity were studied. In the staining of a protein in buffered solution, it was shown that staining intensity (the index of the concentration of the dye-protein compound) at a given pH-value is dependent upon the interaction of the dye-protein, buffer-protein and dye-buffer systems, and that as the dye or buffer or their concentrations were varied, the resultant "isoelectric points" which were obtained also varied. In view of these facts and of the present lack of knowledge of dyes and dye-protein combinations it would be impossible to determine a true isoelectric point by staining at controlled pH-values without further extensive work on the subject. It follows that no true isoelectric points have hitherto been obtained for nucleus, cytoplasm or other tissue elements by staining at controlled pH.**

INTRODUCTION

Since Loeb (1922) showed that it was possible to approximate the isoelectric point of gelatin by staining it with certain acid and basic dyes in a series of buffered solutions, a number of workers have applied this method to determine the "isoelectric points" of the nucleus and cytoplasm of various tissues.

The theory underlying this method of determining isoelectric

[1]From a dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Zoology in the Graduate Division of the University of California.

points is as follows: If the amount of dissociation of a protein or other ampholyte is plotted against pH, a curve will be obtained having two maxima and one minimum. The pH at which the minimum occurs is defined as the isoelectric point of the protein. On the more acid side of the isoelectric point (i.e., at lower pH-values), the protein is dissociated as a positively charged ion. On the other side of the isoelectric point, it is dissociated as a negatively charged ion. Near the isoelectric point, however, there will be a certain small percentage of negative ions on the acid side, and a similar small percentage of positive ions on the basic side, since the dissociation curves of the acid and basic groups of the protein are sigmoid in shape, and the isoelectric point is the point at which the two cross.

Acid dyes dissociate to form negatively charged dye ions. Basic dyes dissociate into positively charged dye ions. Therefore the acid dyes will combine with the positively charged protein ions at pH-values below the isoelectric point, and basic dyes will combine with the negatively charged protein ions at pH-values above the isoelectric point. Assuming the acid dye to be completely dissociated, or nearly so, if a series of dye solutions is set up at different pH-values and samples of a protein are stained in them, the intensity of staining will depend on the amount of ionization of the protein. Hence if intensity of staining is plotted against pH, a sigmoid curve should result, which indicates the amount of dissociation into positive ions of the protein. Similarly, with a basic dye a curve is obtained which indicates the amount of dissociation of the protein into negative ions. The isoelectric point should be the point at which these two curves cross. It will be noted that the above discussion is based on the stoichiometric theory of staining. This is done merely for the sake of convenience, as the adsorptive theory would be equally applicable. For a more detailed discussion than is given here, the paper of Craig and Wilson (1937) may be consulted.

Previous workers, staining tissues as outlined above, have found that with acid dyes the intensity of staining decreases as the pH increases, that with basic dyes the intensity increases with increasing pH, and that the curves obtained are roughly sigmoid. With this confirmation of the theory, they have proceeded to determine isoelectric points for the nucleus and cytoplasm of a wide variety of tissues. None of them, however, has attempted a systematic investigation of the effects of various factors on the isoelectric points obtained. In view of the number of papers on the subject which have been published, and of the significance of the results if valid, it was determined to subject the method itself to a critical evaluation. Fac-

tors which, it was considered, might be important were (1) the dye used, (2) the concentration of dye used, (3) the buffer salts used, and (4) the concentration of buffer used. Each of these was varied independently of the other three, and the results of such treatment noted.

Historical

Pischinger (1926) made the first attempt to determine the isoelectric points of tissue elements. Practically all later workers used his method or a modification of it. He stained gelatin, egg albumin, and alcohol-fixed thymus and cartilage with toluidine blue (a basic dye) and cyanol extra (an acid dye) at a series of pH-values, and plotted the logarithm of the amount of stain against pH. The amount of dye taken up was determined by extracting after staining, and using a colorimeter.

He also stained sections on slides of alcohol-fixed tissues. The slides were stained ten minutes and washed briefly with the proper buffers. The toluidine blue preparations were placed in 4% ammonium molybdate to prevent extraction of stain in alcohol. Most of the water was removed with filter paper, and the slides passed thru xylene-alcohol and xylene to balsam. Stain intensity was estimated by microscopic inspection. Pischinger gives a table of isoelectric points and ranges for the nucleus, cytoplasm and tissue elements of various tissues. These differ for different tissues.

In one case information is given on the effect of buffer concentration on the staining. With toluidine blue, Pischinger found that the neurofibrils stained more intensely and in a more acid region in M/20 buffers than in M/200 buffers. The stain intensity of the former at pH 5.2 was about equal to that of the latter at pH 6.0.

Methyl violet 6B, safranin, Congo red, and picric acid were also used. Pischinger states that the results with safranin and picric acid were in general the same as those for toluidine blue and cyanol extra respectively. With methyl violet, the isoelectric point was shifted toward the acid side. With Congo red, there was a slight decrease in staining on the alkaline side, but even at pH 7.6 (the highest used) the tissues contained a considerable amount of dye. At no pH-value were the nuclei stained, but the dye was precipitated below pH 3.3, so Pischinger could go no lower.

In spite of these contradictory results, Pischinger believed that he had obtained true isoelectric points with toluidine blue and cyanol extra. In a later paper (1927) on the isoelectric points of muscle constituents, he changed his dyes to methylene blue and crystal ponceau.

Other investigators were Pulcher (1927), who worked with smears of frog blood, guinea pig blood, egg albumen, fresh fibrin and "stromi di emazie"; Mommsen (1927), Schwartz-Karsten (1927) and Ochs (1928), who worked with air-dried human blood streaks; Tolstoouhov (1927, 1928), working with blood smears and tissue sections; and Pfeiffer (1929), who worked with plant tissues.

Zieger (1930a, b) continued Pischinger's work by investigating the effect of the fixing fluid on the isoelectric points of a number of tissues. He used M/200 methylene blue and crystal ponceau as his basic and acid dyes. Each fixative gave a different isoelectric point for the same tissue, but with different tissues the shift in isoelectric point as compared with that of absolute alcohol was not invariably in the same direction. Altho, as this paper will show, these so-called "isoelectric points" obtained by Zeiger are not the actual isoelectric points of the tissues, nevertheless his paper is of interest. The relative shifts in isoelectric range are possibly valid.

Others who used similar methods to obtain isoelectric points of various tissues were Yasuzumi (1933a, 1933b, 1934), Nishimura (1934), Yasuzumi and Matsumoto (1936), Sturm (1935), Achard (1935), Fautrez (1936) and Ikeda (1935, 1936).

A lengthier historical account is given by Levine (1937).

Method

The material selected for the investigation was the hypermastigote flagellate, *Trichonympha collaris*. This protozoon is to be found in great abundance in the intestine of the damp-wood termite *Zootermopsis angusticollis*, which is common in the San Francisco Bay area. Thousands of organisms are present in one termite. Coverslip smears were made by removing the hindgut, breaking its wall, and distributing the exuding material as evenly as possible on the coverslip with a pair of dissecting needles. In order to cleanse their intestines of wood particles, whose color would obscure the staining reactions, the termites were previously fed on filter paper for at least a week before use.

Before any drying could take place the smears were dropped into a fixative. Five different fixatives were used, Susa's, Schaudinn's, Bouin's, absolute alcohol, and liquid air. The first three fixatives were used at 60° C., the absolute alcohol at room temperature. Fixation lasted 30 minutes except with liquid air, in which an immersion of 30 seconds was sufficient. Material fixed in liquid air could not be stained at pH-values above 5.0, because the low acidity allowed the protozoa to come off the coverslips. Liquid air fixation

was also unsatisfactory with acid dyes. The protozoa stained readily, but the stain came out quickly in the subsequent washing process. Therefore no isoelectric points could be calculated for this fixative. With this exception, all fixatives were used in all stains.

After fixation the coverslips were washed in running tap water for an hour to remove the fixatives, and then transferred to the staining solutions. After staining for 36 hours, at which time equilibrium was very nearly approached, the smears were washed thoroly in distilled water. Then the coverslips were passed thru four changes of tertiary butyl alcohol and two changes of neutral xylene, and mounted in neutral Canada balsam. The tertiary butyl alcohol prevented the loss of stain which would have taken place in ethyl alcohol. (Levine, 1939).

All staining solutions were prepared in the same way. Dye and buffer solutions of twice the concentration required were made up, and equal quantities of each were mixed to obtain the final staining solutions. The actual pH-values of the solutions were determined by means of a glass electrode. The addition of dye changed the pH-values of the buffers, the amount of change depending on the dye and on its concentration. In order to obtain buffer or dye solutions of different concentrations, the concentrations of the stock solutions were varied, or the buffered solutions were diluted as required.

The following series were run:

1. The effect of different dyes on the "isoelectric point" obtained.

Fixatives:—Susa's, Schaudinn's, Bouin's, absolute alcohol, liquid air.

Buffer:—McIlvaine's citric-acid–secondary-sodium-phosphate buffers diluted ten times (referred to hereinafter as 1/10 McIlvaine's buffers), at approximate pH values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0.

Dyes:—Basic dyes—0.0001 *M*[2] toluidine blue O (National Aniline Co., Cert. No. NU-2, dye content 60%), 0.0001 *M* methylene blue U. S. P. medicinal (National Aniline Co., Cert. No. NA-48, dye content 89%), 0.0001 *M* Nile blue sulfate (National Aniline Co., Cert. No. NNb-1, dye content not given), and 0.0001 *M* crystal violet (A. H. Thomas Co., no certification number or dye content given).

Acid dyes—S/20[3] ponceau 2R (Coleman and Bell Co., no certification number or dye content given), and 0.0001 *M* orange G (National Aniline Co., Cert. No. NO-3, dye content 85%).

[2]The concentrations of dye solutions were made on the basis of the dye contents of the dry dyes, except in the cases of Nile blue sulfate, crystal violet and ponceau 2R, for which dye content was not known. In these cases it was arbitrarily assumed to be 80%.

[3]When a 0.002 *M* stock solution of ponceau 2R was made up, it was found that the dye did not all dissolve even after several days. This solution was then filtered, and the filtrate considered a saturated solution. The symbol S/20 thus indicates a 1/20 saturated solution.

2. The effect of concentration of dye on the isoelectric point obtained.

Fixatives:—As above.

Buffer:—As above.

Dyes:—The same six dyes were used as above, in concentrations of 0.0001 *M*, 0.00025 *M*, 0.0005 *M*, and 0.001 *M*. In the case of ponceau 2R the concentrations were S/20, S/8, S/4, and S/2.

3. The effect of different buffers on the isoelectric point obtained.

Fixatives:—As above.

Buffers:—All buffers were diluted to one-tenth their standard strength.

(1) 1/10 McIlvaine's buffers (citric acid and secondary sodium phosphate) at pH-values approximately 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0.

(2) 1/10 Sôrenson's buffers (sodium citrate and HCl) at pH-values approximately 1.0, 2.0, 3.0, 4.0 and 5.0.

(3) 1/10 Sôrensen's buffers (primary potassium phosphate and secondary sodium phosphate) at pH-values approximately 5.0, 6.0, 7.0 and 8.0.

(4) 1/10 Clark and Lubs' buffers (potassium acid phthalate and HCl) at pH-values approximately 3.0 and 4.0.

(5) 1/10 Clark and Lubs' buffers (potassium acid phthalate and NaOH) at pH-values approximately 5.0, 6.0 and 7.0.

Dyes:—(1) 0.0001 *M* toluidine blue O.
(2) S/20 ponceau 2R.

4. The effect of different concentrations of buffer on the isoelectric point obtained.

Fixatives: As above.

Buffers:—The same buffers were used as in the preceding series. McIlvaine's and Sorensen's citrate-HCl buffers were used in standard, 1/10 standa d, and 1/100 standard concentrations, the other buffers in standard and 1/10 standard concentrations.

Dyes:—(1) 0.0001 *M* toluidine blue O.
(2) S/20 ponceau 2R.

To determine intensity of staining, color charts were prepared as follows: A series of eight small squares was ruled off on white drawing paper. The first square was painted evenly with a 1% solution of one of the dyes, the second square with a 0.5% solution, the third with a 0.25% solution, and so on down to the last square, which was faintly colored by the 1/128% dye solution. Thus a geometric progression in intensity was obtained, each step being double the intensity of the preceding one. These charts had approximately the same shade of color as the slides, except in the case of crystal violet, in which the stained organism contained more red than did the color chart. The difference was not sufficient to interfere with color comparison, however. With the aid of these charts, the intensity of staining of the *Trichonympha* nucleus and cytoplasm under the various experimental conditions was recorded. The methylene blue chart was used on

organisms stained in Nile blue sulfate and on the nuclei stained in toluidine blue O, since these dyes gave its color and not those of their own charts. While this method involves the use of the eye in comparing intensities, it was found that after some practice a high degree of accuracy was obtained as judged by the results of repeated determinations made on the same material at different times.

Results

The experimental values obtained were plotted on graph paper with stain intensity on the ordinate, and pH on the abscissa. From these graphs, "isoelectric points" were obtained and tabulated. In

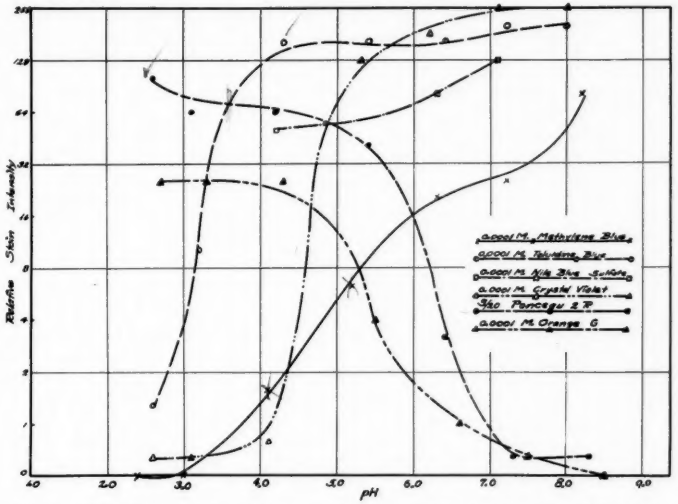

Fig. 1. The effect of different dyes on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1–10.

all, 136 graphs were constructed and used, but it is obviously impracticable to present more than a few representative ones in this paper. All the other graphs are to be found in the writer's doctorate thesis (Levine, 1937).

Series 1. The effect of different dyes on the "isoelectric point" obtained.

In Fig. 1 stain intensity is plotted against pH for the six dyes used with *Trichonympha collaris* nucleus after Susa's fixation.

Theoretically, if the curves determine isoelectric point, those of the four basic dyes should all be the same curve or approximately the

same, and those of the two acid dyes should be the same. The pH-value at which these two ideal curves crossed would be the isoelectric point of the cell element. This is obviously far from the fact.

Table 1 gives the "isoelectric points" obtained from the intersection of the acid and basic curves for the nucleus of *Trichonympha collaris*. With any given fixative, the isoelectric point should be the same regardless of the dyes used in obtaining it. Therefore all the figures in each vertical column of each table should be the same. Far from this, Table 1 gives values for the nucleus fixed in Susa's ranging from 3.3 to 6.0, with Schaudinn's from less than 2.6 to 4.9, with Bouin's from 3.8 to 5.6, and with absolute alcohol from much less than 2.6 to 4.8. Similar results were obtained for the cytoplasm.

TABLE 1. "ISOELECTRIC POINTS" OF TRICHONYMPHA COLLARIS NUCLEUS

Dye Combination*	Fixative			
	Susa's	Schaudinn's	Bouin's	100% Alcohol
P—TB	3.6	3.5	4.7	3.3
OG—TB	3.3	3.0	4.2	3.0
P—CV	4.9	3.0	4.7	<<2.6*
OG—CV	4.7	2.9	4.5	<<2.6
P—NBS	4.9	3.1	4.7	<2.6
OG—NBS	<4.2	<2.6	3.8	<2.6
P—MB	6.0	4.9	5.6	4.8
OG—MB	5.3	3.9	5.3	4.3

*The symbols used in the table are:
P = Ponceau 2R
OG = Orange G
TB = Toluidine blue O
CV = Crystal violet
NBS = Nile blue sulfate
MB = Methylene blue
<< = "much less than"

Even if the results with only one acid dye are used, the range becomes only a little narrower. For instance with ponceau 2R as the acid dye, the isoelectric points of the nucleus range with Susa's fixation from 3.6 to 6.0, with Schaudinn's from 3.0 to 4.9, with Bouin's from 4.7 to 5.6, with absolute alcohol from much less than 2.6 to 4.8. Similar results are obtained with the cytoplasm, or with orange G substituted for ponceau. With this assortment of "isoelectric points," it would seem difficult to pick the correct one, if any is correct.

Certain facts appear from the data. First, orange G gives a consistently lower "isoelectric point" with any of the basic dyes than does ponceau 2R. Second, methylene blue gives a consistently higher "isoelectric point" with either of the acid dyes than does any other basic dye. The other three basic dyes do not arrange them-

selves in any consistent consecutive order. Third, when the fixation is the same, the "isoelectric points" of the cytoplasm are consistently higher than those of the nucleus. This is in accord with the results of Pischinger (1926, 1927), Zeiger (1930 a, b), Craig, and Wilson (1937), etc.

SERIES 2. THE EFFECT OF CONCENTRATION OF DYE ON THE ISOELECTRIC POINT OBTAINED.

In Fig. 2 stain intensity is plotted against pH for different concentrations of the same dye. The results obtained on *Trichonympha collaris* nucleus fixed in Bouin's and stained with toluidine blue O are shown. The curves obtained with the other combinations of dyes and fixatives are essentially similar in their variability.

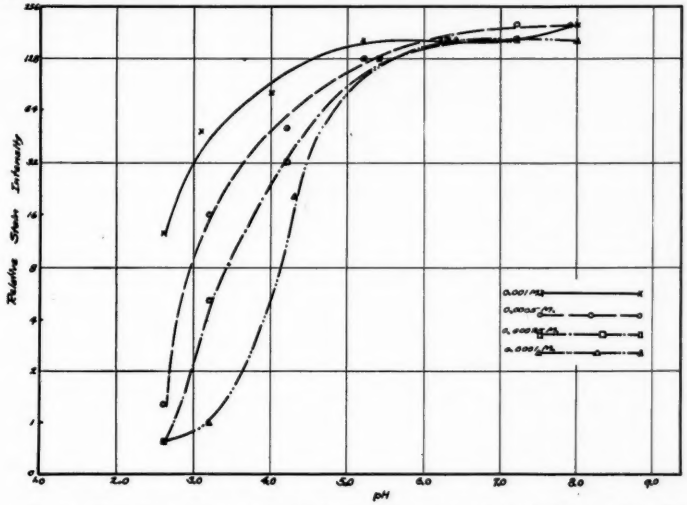

Fig. 2. The effect of dye concentration on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Bouin's. Dye—toluidine blue O. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1–10.

An analysis of these graphs shows that different concentrations of dye give different curves, which may be more or less widely separated. Usually, the higher the concentration of basic dye, the further to the acid side is the resultant curve, and the lower would be the "isoelectric point" obtained by its use. With acid dyes, the higher the concentration of dye, the further to the basic side is the resultant curve, and the higher would be the "isoelectric point" obtained; this point is illustrated in Fig. 3. These two opposite tendencies cancel each other out in part, but there is still a great discrepancy in the

resultant isoelectric points. In Table 1 are given the "isoelectric points" obtained for Schaudinn-fixed *Trichonympha* nucleus by superposition of the acid and basic dye curves. The degree of variability of "isoelectric points" of nucleus and cytoplasm with the other fixatives was essentially similar.

It is apparent that as the concentration of acid dye decreases, the pH of the "isoelectric point" also tends to decrease, and as the concentration of basic dye decreases, the pH of the "isoelectric point" tends to increase. This general rule has many exceptions, of which a large number are due to the positions of the points of intersection of

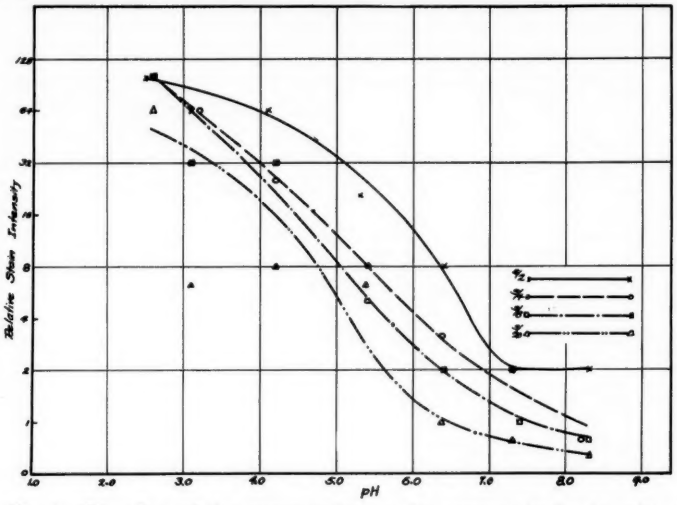

Fig. 3. The effect of dye concentration on the apparent isoelectric point of *Trichonympha collaris* cytoplasm. Fixative—Schaudinn's. Dye—ponceau 2R; dilution symbols (S/2, S/4, etc.), indicate degree of saturation. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1–10.

the acid and basic curves. If a true isoelectric point were being obtained, the two curves should intersect near their lower ends, for the isoelectric point represents a minimum amount of dissociation. But this is seldom the case. Most intersections occur near the upper extremities of one or both curves, some near the midpoint, and few below the midpoint. This is another point indicative that the true isoelectric point is not obtained.

SERIES 3. THE EFFECT OF DIFFERENT BUFFERS ON THE ISOELECTRIC POINT OBTAINED.

In figure 4 are shown the curves obtained with 0.0001 *M* toluidine blue O on Susa-fixed *Trichonympha collaris* nucleus. The results

TABLE 2. THE EFFECT OF DYE CONCENTRATION ON THE "ISOELECTRIC POINT" OF SCHAUDINN-FIXED TRICHONYMPHA COLLARIS NUCLEUS

Acid Dye	Basic Dye: Crystal Violet				Basic Dye: Toluidine Blue O			
	0.001 *M*	0.0005 *M*	0.00025 *M*	0.0001 *M*	0.001 *M*	0.0005 *M*	0.00025 *M*	0.0001 *M*
Ponceau 2R								
S/2*	2.3	2.5	2.6	3.4	3.0	3.3	3.6	3.9
S/4*	2.6	2.7	2.7	3.3	3.0	3.2	3.5	3.8
S/8*	2.4	2.6	2.6	3.3	3.0	3.2	3.5	3.8
S/20*	<2.4	<2.5	<2.6	3.1	2.8	2.9	3.3	3.6
Orange G								
0.001 *M*	<2.4	<2.5	<2.6	2.7	<2.6	<2.6	2.6	3.2
0.0005 *M*	<2.4	<2.5	<2.6	2.8	<2.6	<2.6	2.6	3.3
0.00025 *M*	<2.4	<2.5	<2.6	2.8	<2.6	<2.6	2.8	3.3
0.0001 *M*	<2.4	<2.5	<2.6	2.7	<2.6	<2.6	<2.6	3.0

Acid Dye	Basic Dye: Nile Blue Sulfate				Basic Dye: Methylene Blue			
	0.001 *M*	0.0005 *M*	0.00025 *M*	0.0001 *M*	0.001 *M*	0.0005 *M*	0.00025 *M*	0.0001 *M*
Ponceau 2R								
S/2*	2.8	3.4	3.5	3.7	5.1	5.0	5.2	5.7
S/4*	2.6	3.3	3.4	3.6	4.9	4.8	5.0	5.4
S/8*	2.6	3.3	3.4	3.6	4.9	4.7	4.9	5.3
S/20*	<2.4	3.0	3.1	3.4	4.8	4.5	4.7	5.0
Orange G								
0.001 *M*	<2.4	<2.5	<2.6	<2.6	3.1	3.7	4.1	4.5
0.0005 *M*	<2.4	<2.5	<2.6	<2.6	3.4	3.9	4.2	4.6
0.00025 *M*	<2.4	<2.5	<2.6	<2.6	3.7	4.0	4.2	4.6
0.0001 *M*	<2.4	<2.5	<2.6	<2.6	2.6	2.9	3.5	3.8

*S/2=half saturated solution, S/4=quarter saturated solution, etc.

obtained with the other fixatives and with ponceau 2R are similar. It is seen that at a given pH the same stain intensity is not obtained with different buffers. Table 3 gives the "isoelectric points" of *Trichonympha* nucleus under different conditions of fixation and buffering. The isoelectric points were determined by superimposing the curves for ponceau 2R on those for toluidine blue O. With the nucleus, the range of "isoelectric points" obtained with the same fixative covers from 0.9 pH-units (Bouin's) to 1.7 pH-units (Susa's). A similar variation was observed with the cytoplasm. The buffers can be arranged in a regular series with regard to their effect on the

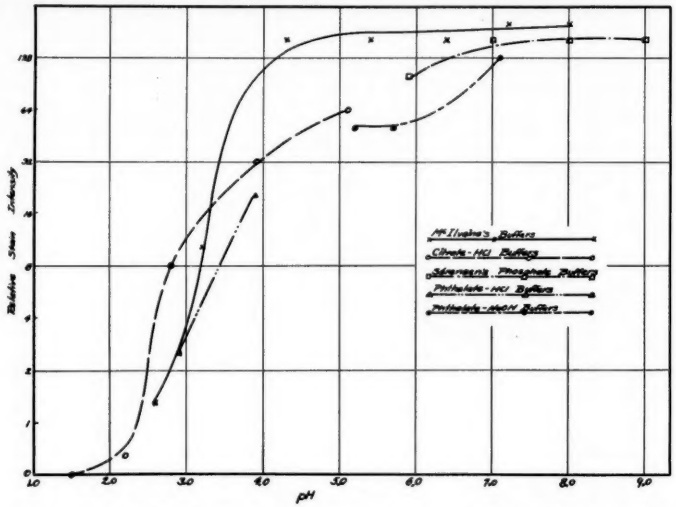

Fig. 4. The effect of different buffers on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. Dye—toluidine blue O. All buffers 1/10 standard concentration.

"isoelectric point." McIlvaine's buffers (citric acid and secondary sodium phosphate) give the lowest isoelectric points; Sörensen's buffers (sodium citrate and HCl) give higher isoelectric points; and Clark and Lubs' buffers (potassium acid phthalate and NaOH) give still higher ones. If the other two buffers had covered a wide enough pH range, they would undoubtedly have given still different "isoelectric points."

SERIES 4. THE EFFECT OF THE CONCENTRATION OF BUFFER ON THE ISOELECTRIC POINT OBTAINED.

Figure 5 shows the effect of the concentration of McIlvaine's buffer on the isoelectric point of *Trichonympha* nucleus, when

stained with toluidine blue O after fixation in Susa's. A similar variability was obtained with the other fixatives, with ponceau 2R and with the other buffers.

TABLE 3. THE EFFECT OF DIFFERENT BUFFERS ON THE "ISOELECTRIC POINT" OF TRICHONYMPHA COLLARIS NUCLEUS

Fixative	Buffer		
	McIlvaine's	Citrate-HCl	Phthalate-NaOH
Susa's	3.7	4.6	5.4
Schaudinn's	3.6	3.9	<5.2
Bouin's	4.7	4.5	5.6
Alcohol	3.4	3.7	<5.2

The accuracy of the very dilute buffer curves (i.e., 1/100 the original concentration) is doubtful. It is very probable that in such dilute systems the reactions changed in the course of the staining process. If these are omitted from consideration, it is seen that as a general

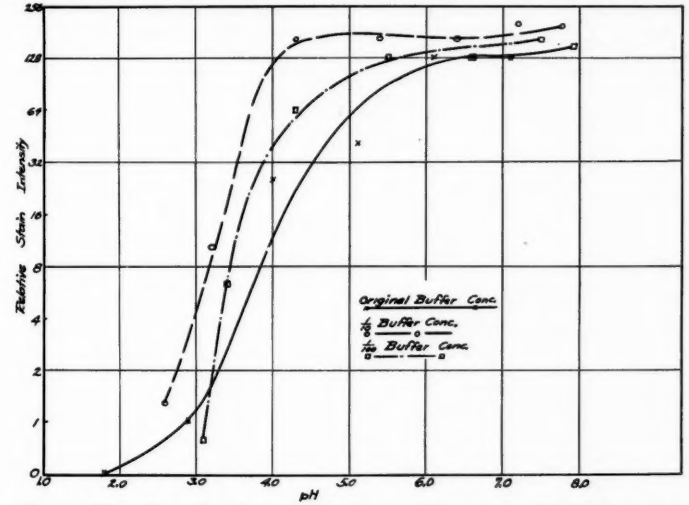

Fig. 5. The effect of buffer concentration on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. Dye—toluidine blue O. McIlvaine's buffers (citric acid and secondary sodium phosphate).

rule the more concentrated the buffer, the less intense is the staining at any given pH-value.

Table 4 gives for *Trichonympha* nucleus the isoelectric points obtained from the graphs by superimposing the ponceau curves on the toluidine blue ones. The lowered staining intensity with increased buffer concentration would tend to produce a higher iso-

electric point with toluidine blue and a lower one with ponceau. These two opposite effects partially cancel each other out, but as a result the isoelectric points obtained have no constant relation to each other.

STAINING OF CASEIN

In order to check the experiments on *Trichonympha*, casein was stained at different pH-values with toluidine blue O and methylene blue. The casein, prepared by the method of Van Slyke and Baker and washed thirty days, was obtained from Dr. P. L. Kirk of the Department of Biochemistry, University of California. One-tenth-gram samples were placed in 10 cc. of 0.0003 *M* dye made up in 1/10 McIlvaine's buffers (citric acid and secondary sodium phosphate) at pH values approximately 2.2, 3, 4, 5, 6, 7, and 8. The relative amounts of stain taken out of solution by the protein at different reactions was noted after 24, 48, and 72 hours. There was no essential difference between the results at these times.

TABLE 4. EFFECT OF BUFFER CONCENTRATION ON THE "ISOELECTRIC POINT" OF TRICHONYMPHA COLLARIS NUCLEUS

Buffer	Fixative			
	Susa's	Schaudinn's	Bouin's	Alcohol
McIlvaine's				
Standard Conc.	4.4	3.3	4.3	2.8
1/10 Conc.	3.7	3.4	4.3	3.3
1/100 Conc.	3.7	3.7	4.2	3.8
Citrate-HCl				
Standard Conc.	>4.7	3.6	>4.7	3.1
1/10 Conc.	4.7	3.9	4.6	3.7
1/100 Conc.	4.7	3.8	4.4	3.5
Phthalate-NaOH				
Standard Conc.	6.5	5.7	5.9	<5.0
1/10 Conc.	5.4	<5.2	5.6	<5.2

At pH 6, some protein was dissolved; at pH 7 most of it was dissolved, and at pH 8 all the casein went into solution. These three pH-values were therefore valueless, since it was impossible to determine how much dye was combined with the protein, and how much was not. At any lower pH-value, however, it was found that relatively more methylene blue than toluidine blue was present in the supernatant fluid above the dye-protein precipitate. Therefore methylene-blue-casein is more completely ionized at these reactions than is toluidine-blue-casein. Hence the intensity of staining of casein is less with methylene blue than with toluidine blue. This result is in complete accord with the results obtained for *Trichonympha*.

Discussion

In order to apply a staining method to the determination of isoelectric point, the cells must be killed and fixed. Unless the cell is dead, most dyes will not penetrate its outer membrane. Therefore in no case does the method establish the isoelectric points of the living proteins, but only those of the proteins of the dead organism. Different fixatives, as Zeiger (1930b) has shown, give different isoelectric points. It has been assumed that absolute alcohol fixation does not alter the isoelectric points of tissue proteins. It is possible, however, that alcohol does in fact shift the isoelectric point. Certainly it changes the nature of the proteins markedly.

Except in the case of blood cells, it has been necessary to section the tissues before they could be stained. Before sectioning, the fixed block of tissue must first be passed thru increasing grades of alcohol, xylene or toluene, and paraffin. Then, after mounting, the sections must again be passed thru xylene and alcohol to water before staining. This treatment undoubtedly has some effect on the proteins. The resultant effect is not that of the fixative alone. The effect of a post-fixation in alcohol, xylene, paraffin, etc., is added. The degree to which this may be a disturbing factor is not known. In the present investigation the necessity of sectioning was eliminated by the use of protozoan smears.

In all work reported on blood streaks, the streaks are air-dried before staining. Air-drying, as Seki (1934) has pointed out, makes the cell membranes more impermeable to staining solutions, and also has the more serious effect of markedly altering the "isoelectric points" of the cell proteins. The experiments in which this took place are, however, of value with respect to the development of a practical, pH-controlled blood stain.

Most of the investigations of "isoelectric point" have utilized concentrated dye solutions and a short staining period. A ten-minute immersion was the usual time, and equilibrium was not reached. This treatment introduces another unknown variable into the reaction, i.e., the rate of staining. Its elimination would be desirable. This can easily be accomplished by using dilute staining solutions, and allowing the reaction to proceed to equilibrium, as was done in the present work.

When the slides are run up thru alcohol before mounting, there is always a loss of stain in the alcohol. This is particularly serious in the case of methylene blue and toluidine blue, the basic dyes used by most investigators. Such a loss of stain would result in throwing the results far off. In order to prevent it as much as possible, slides

stained in these two dyes were fixed by these investigators in ammonium molybdate solution before being passed into alcohol. Altho this treatment is helpful, it nevertheless allows a great deal of dye to escape. For this reason, results previously reported with these two dyes should be viewed with some suspicion. Levine (1939), however, found that the substitution of tertiary butyl alcohol for ethyl alcohol in the dehydration process eliminated practically all loss of stain. This technic was used in the present work, and ammonium molybdate fixation was not found to be necessary.

To obtain the isoelectric point of a protein from its staining properties, the intensity of staining with an acid and a basic dye should be plotted against pH. Two opposite curves will be obtained, and the isoelectric point is theoretically the pH-value at which the curves cross. This is the basis on which Yasuzumi obtained his isoelectric points. Pischinger, Zeiger, etc., however, used only the curves for their basic dyes, and considered the isoelectric points to be the midpoints of these curves. This assumption is not valid.

Many investigators have not determined the pH-values of their staining solutions directly, but obtained the pH-values of their buffers colorimetrically and assumed that the addition of dye did not alter the reaction appreciably. It is a fact, however, that the addition of dye, particularly of as much dye as ordinarily used, may shift the reaction several tenths of a pH-unit. The glass electrode is the most satisfactory instrument for determining the pH of dye solutions, since hydrogen and quinhydrone electrodes are liable to be poisoned either by the dye itself or by unknown impurities present in it, and since the glass electrode has no tendency to drift. In the present investigation a glass electrode was used to determine the pH-values of the buffered staining solutions.

When a dye stains a protein, the intensity of staining is an index of the amount of dye-protein compound formed. According to the mass law, the amount of dye-protein compound is dependent on the concentrations of dye and protein ions in the reacting system. The concentration of dye ion is a function of the amount of dye present, its dissociation constant, and of pH. Similarly, the concentration of protein ion is a function of the amount of protein present, the isoelectric point of the protein, and the pH of the solution. If, then, dyes are chosen with sufficiently great dissociation constants to be completely ionized, or nearly so, over the pH-range in which they are used, at first glance it would appear possible to determine the isoelectric point of a protein by staining at varying pH-values.

Unfortunately, it is not such a simple matter. The solubility

product or dissociation constant of the dye-protein compound must be taken into consideration. This determines what proportion of the dye-protein compound itself is ionized at any pH-value. An example will show the importance of this factor. Take, for instance, two dye-protein compounds, AP and BP, in which the protein is the same but the dyes are different. At a given pH-value the original concentration of protein ions is the same for both systems. If the dyes selected are completely dissociated at this reaction (they can be selected so that they will be) and if the dye concentrations are the same, the amount of dye-protein compound formed will be the same in both cases. But if the dissociation constant of AP is greater than that of BP, at any pH-value below that at which both are completely dissociated and above that at which neither is dissociated, AP will be more dissociated than BP. In other words, more dye will be ionized from AP than from BP, and consequently the actual concentration of undissociated dye-protein compound will be less for AP than for BP. Hence, the resultant intensity of staining will be less with dye A than with dye B at the same pH. Hence when staining intensity is plotted against pH for the two dyes, different curves will result. Then if one plots curves for two basic dyes and one acid dye, and takes the isoelectric point as the pH at which the acid and basic dye curves cross, two different isoelectric points will be obtained. The more dyes used, the greater the number of "isoelectric points" which will be obtained. The use of different fixatives changes the nature of the proteins, introducing new isoelectric points and new dye-protein dissociation constants. This theoretical reasoning is confirmed by the experimental results obtained in the staining of casein with methylene blue and toluidine blue O (see p. 103), and also by the experimental results given in Table 1. The "isoelectric points" obtained with the different dye combinations range over more than two pH units. This fundamental deficiency of the method makes it impossible to obtain true isoelectric points by its use. No criterion is available to establish which particular combination of dyes gives a true isoelectric point, if any do.

Further confirmation is obtained from Fig. 1 in the paper of Rawlins and Schmidt (1929). Here are plotted titration curves of casein against methylene blue, safranin Y and indulin scarlet for pH-values from 6 to 12. In their experiments pH was established with HCl or NaOH, and not with buffers, and the dye-protein compounds were insoluble even at the high pH-values. The amount of combination was determined colorimetrically. From pH 7 to 12 the amount of dye-protein for the three dyes was approximately the

same. This is to be expected on the basis of the theory. At pH 6, however, the three curves separate. The casein binds 100×10^{-5} equivalents of methylene blue, 150×10^{-5} equivalents of indulin scarlet, and 175×10^{-5} equivalents of safranin Y per gram of protein. Had the curves been carried to a lower pH-value, the results would have been even more striking.

It follows from the mass law that the lower the dye concentration, the less dye-protein compound will be formed at any reaction. Hence, when different concentrations of dye are used, the curves for staining intensity plotted against pH should be different. With basic dyes, as concentration of dye decreases, the curve will be shifted toward a higher pH-value. With acid dyes, the curve will be shifted the other way. As a result, different "isoelectric points" will be obtained with different dye concentrations. A glance at Fig. 2 and Table 2 will show that even with the complex mixture of proteins in protoplasm the above prediction is confirmed.

The buffer system also has its effect on the staining curves. This is because the amount of dye-protein compound is dependent on the concentrations of ionized dye and protein which are free to combine at any reaction. The buffer salts are not inert, but react with both dye and protein. They bind a certain amount of both in the form of un-ionized dye-buffer and buffer-protein compounds. Hence the amount of free dye and protein ions is decreased by the buffer salts. The extent to which this takes place depends on the dissociation constants of the dye-buffer and buffer-protein compounds. The reasoning is similar to that employed for the dye-protein reaction. The smaller the dissociation constant of the dye-buffer compound, the more dye is inactivated by the buffer, and the less will be the quantity of dye-protein compound resulting. Similarly, the smaller the dissociation constant of the buffer-protein compound, the more protein will be inactivated by the buffer, and the less protein will be free to combine with the dye.

The dissociation constants of the dye-buffer and buffer-protein compounds depend in part on those of the buffer salts employed. Hence it would be expected that with different buffers, these dissociation constants would be different and that different amounts of dye and protein would be inactivated. Therefore when staining intensity is plotted against pH, different curves would result with different buffer systems. This is the case in the experiments described in the present paper. "Isoelectric points" almost two pH-units apart were obtained simply by varying the buffer system.

According to the mass law, if the concentration of buffer salts be

increased, more dye and protein should be taken up by the buffer system, and hence less dye-protein compound would be formed. Consequently, with basic dyes, the more concentrated the buffer, the further to the alkaline side the curve of staining intensity will be shifted. For acid dyes, with the more concentrated buffer, the curve will be shifted to the acid side. The experimental results of the present investigation show that this relation holds in the staining of protoplasm.

Temperature, too, plays a part in the reaction, since it changes the dissociation constants of the various compounds present. In short, if the temperature factor is ignored, the intensity of staining depends at a given pH-value upon the interaction of three systems, the dye-protein, dye-buffer, and buffer-protein. If any one factor changes, the intensity of staining also changes.

Any attempt to determine the isoelectric point of a protein by a staining method in the present state of our knowledge would be useless. Even if the dissociation constant of the dye were known, or if it were high enough to produce complete dissociation over the pH-range studied, the isoelectric point would be one of three unknown factors, the other two being the dissociation constants of the dye-protein and the buffer-protein compounds. These would depend in part on the dye and the buffer salts utilized, and therefore when these were varied the results would not be the same.

The above discussion applies to pure proteins. It should be emphasized that protoplasm is not a pure protein, but an extremely complex system of many kinds of proteins, carbohydrates, lipoids and salts. Its protein complex has no true isoelectric point, but rather a more or less broad isoelectric range which is the expression of the isoelectric points of the component proteins. Then, too, the lipoids, carbohydrates and salts are not inert. Reactions between certain dyes and certain of these other constituents would give misleading results. Undoubtedly such reactions occur. It is known that alizarin red S has a strong affinity for calcium. Craig has found (oral communication) that malachite green is precipitated by the phosphate ion. Both calcium and phosphate are always found in protoplasm. Any dye used to determine isoelectric point may, for all we know, have a similar reaction with some specific non-protein ion present in the protoplasm. In such a case it would be expected that different dyes would give different staining results. While the results obtained by Pischinger, Zeiger, the writer and others indicate that cytoplasm and nucleus have different isoelectric ranges and are composed of proteins whose isoelectric points are fairly close to each

other, little more can be said. Since it is at present impossible to find the true isoelectric point of a single protein by the staining method, it would be useless to try to use it to determine actual isoelectric ranges of protoplasmic elements.

Summary

1. The staining reactions at controlled pH-values of various dyes with the nucleus and cytoplasm of *Trichonympha collaris* under different conditions have been investigated. When stain intensity was plotted against pH it was found that:

a) Different curves were obtained for the acid dyes, ponceau 2R and orange G, from those obtained with the basic dyes, toluidine blue O, methylene blue, Nile blue sulfate, and crystal violet.

b) When the concentrations of ponceau 2R and toluidine blue O were varied, the intensity of staining at any pH was a direct function of the concentration of dye.

c) When staining in toluidine blue O and ponceau 2R, stain intensity at a given pH-value was found to be a function of the buffer system in which the staining took place. "Isoelectric points" obtained by superposition of the two dye curves were higher in Sörensen's buffers (sodium citrate and HCl) than in McIlvaine's (citric acid and secondary sodium phosphate), and still higher in Clark and Lubs' (potassium acid phthalate and NaOH).

d) When the concentration of buffer was increased, a lower intensity of staining was obtained than with a more dilute buffer.

2. Various factors modifying staining intensity are discussed from a theoretical point of view. In the staining of a protein in buffered solution, it was shown that staining intensity (the index of the concentration of the dye-protein compound) at a given pH-value is dependent upon the interaction of the dye-protein, buffer-protein and dye-buffer systems, and that as the dye or buffer or their concentrations were varied, the resultant isoelectric points which were obtained also varied. In view of the present lack of knowledge of dyes and dye-protein combinations it would be impossible to determine a true isoelectric point by staining at controlled pH-values without further extensive work on the subject. It follows that the method cannot yet be applied to such a complex system as protoplasm, the aggregate of whose proteins have a more or less broad isoelectric range rather than a true isoelectric point, and which is by no means purely protein in nature.

3. No true isoelectric ranges have hitherto been obtained for nucleus, cytoplasm or other tissue elements by staining at controlled pH-values.

ACKNOWLEDGMENTS

I should like here to thank Dr. Roderick Craig for his helpful advice in the performance of the present research, Dr. Paul L. Kirk for furnishing the pure casein used in this research, and my wife, Helen Saxon Levine, for her assistance in the preparation of this paper.

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A METHOD FOR STAMPING SERIAL NUMBERS ON CELLOIDIN SECTIONS

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One of the best methods of maintaining celloidin sections in serial order is the placement of numbers on the sections. This is a very useful procedure whenever removal of the celloidin is not necessary before mounting as it is relatively simple by comparison with other methods.[1] Furthermore, it allows storing and staining of the sections in bulk without loss of serial identity.

This method as first used by Suzuki[2] and later by Da Fano[3] involves writing serial numbers on the celloidin sections with a fine pointed brush after first cutting and arranging them in sequence on a flat surface. The former used India ink as a writing medium while the latter added ether and acetone to improve the writing quality of the ink.

The procedure to be described facilitates the numbering process by simply stamping the figures on the celloidin with an appropriate ink prior to cutting the sections. This eliminates the task of arranging sections in sequence before marking and in addition the numeration is executed more efficiently with a stamp than a brush. During the past several years more than 10,000 sections of Weigert and Nissl preparations have been handled successfully in this manner.

Success depends largely on employing a stamp ink which is readily absorbed by the celloidin, dries quickly and sufficiently resists fading by alcohols and other reagents. A commercial product bearing the trade name of Triune Opaque Stamp Ink (black)[4] has been found to satisfy these requirements. Rotating rubber number stamps to fit most any need may be procured thru a local stamp dealer. We have,

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[1]Reference is made to general accepted methods such as described in Mallory's Pathological Technique, pp. 61–64. Saunders, 1938.

[2]Suzuki, B. 1909. Eine einfache Schnittserienmethode bei der Celloidineinbettung. Anat. Anz., **34**, 358–61.

[3]Da Fano, C. 1925. On the numbering in series of sections from celloidin blocks. Proc. Physiol. Soc., J. Physiol., **60**, 13.

[4]Triune and Trojan stamp inks are manufactured by the Superior Type Company, 1900 W. Larchmont Avenue, Chicago, Ill. In case this product cannot be obtained locally the company will supply the name of the nearest dealer upon request. The usual price for a two oz. bottle is 55 cents. There may be similar inks made by other companies which would serve equally well.

however, used a dime store stamp after first removing the year and month bands. This provides numbers 1 to 99 which suffices in most cases since even large series of over 2,000 sections can be conveniently handled in groups of 99 or less.

The ink dries rather rapidly; therefore, it cannot be used on ordinary stamp pads in the same manner as the slower drying inks. The manufacturer recommends a special one (Opaque Stamp Pad) which reduces evaporation and consequent gumminess. A regular (uninked) pad, however, may be substituted if repeated small quantities of ink are applied as needed.

Method: An adequate margin of celloidin must be provided for placement of the numbers while embedding and trimming the block. Good imprints are obtained by keeping the stamp clean and applying the proper amount of ink and pressure. The numbered section is unrolled on the knife just before completing the cut. After completing the cut, the section is floated on water (contained in a wide shallow dish) with the inked surface upward, thus allowing the ink to dry before storing in 70% alcohol. In case only a single section is to be placed on a slide the sections may be stained and mounted without regard to their sequence. If more than one section is to be mounted on a slide, the desired sections must be selected before mounting. The slides may be easily arranged in proper order after placing them against a white background to offer contrast to the numbers on the celloidin. The slides may then be given corresponding numbers and appropriate labels. It has been found desirable to write or stamp on the slides with Trojan[4] ink as it has excellent writing and wearing qualities on glass.

A METHOD FOR INJECTING INSECT TRACHEAE PERMANENTLY

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ABSTRACT.—**By the use of an alcohol insoluble dye (trypan blue), acetic acid, and a detergent ("Santomerse No. 3"), a resulting dye solution is obtained which will completely penetrate the tracheal system of an insect. The dye is injected by the use of a vacuum and by the pressure produced when the air is allowed to re-enter the dye vessel. The dye itself is permanently fixed in the tracheae by means of a fixing solution containing alcohol, acetic acid and barium chloride as its components. The material must be properly preserved after staining. It may be stored indefinitely in 70% alcohol, xylol, cedar oil or clove oil, depending on whether the material is to be used for sectioning or for whole mounts. Injected material may be sectioned in either celloidin or paraffin, or may be cleared and mounted *in toto*.**

At present there are only two ways of studying the tracheal system of an insect. One is to cut open the live specimen and examine the air-filled tracheae. The other method is to inject a colored oil into the tracheal system to facilitate observation. In each case the material must be used immediately as both air and oil disperse from the tracheae rapidly. Photographs of uncleared and unmounted material are unsatisfactory, and as permanent staining was not possible prior to this study, drawings were necessarily the only permanent record of research done on the tracheal system.

In the method described below, the injected material may be stored an indefinite length of time after fixation in 70% alcohol, xylol, cedar oil or clove oil, depending on whether the material is to be used for sectioning or for whole mounts. Drawings are not necessary as photographs may easily be made of the slides, which are themselves a fixed record.

To inject insect tracheae permanently the following solutions are required:

Solution A—the dye solution,

Trypan blue[1]	2.0g.
Santomerse No. 3[2]	1.0g.
Glacial acetic acid	10 cc.
Distilled water	90 cc.

[1]Trypan Blue—National Aniline and Chemical Co. Inc. C. I. No. 477. Lot No. 7286.

[2]Santomerse No. 3—A detergent produced by the Monsanto Chemical Co., St. Louis, Mo.

Solution B—the fixing solution,

Formaldehyde (40%)	15 cc.
Glacial acetic acid	10 cc.
Saturated solution of barium chloride in distilled water	75 cc.

Live specimens are chloroformed and placed in a small wire basket. The basket is suspended above the stain by means of a fine wire. This wire is inserted into the aperture of the stopcock, which is then

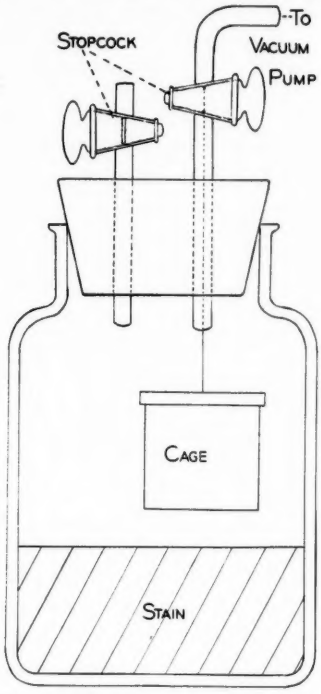

Fig. 1. Diagram of apparatus used to inject stain into insect material.

half closed, pinching the wire and thus holding the basket in place. The air is then exhausted from the vessel containing the stain by means of a vacuum pump. If the water pressure is good a faucet suction pump may be used to create the vacuum. In either case check must be made on the reduction of pressure. The manometer reading should in no case be less than 29 in. of mercury.

After 15 or 20 minutes the basket is dropped into the liquid by opening the stopcock and releasing the wire. The vacuum is maintained for 5 minutes longer and then the air is allowed to re-enter

the vessel slowly thru the other stopcock. (Fig. 1). The specimens are left submerged in the solution until the dye has completely penetrated the tracheae. This requires about 15 minutes.

Upon removal from the dye solution the material is fixed in solution B. Large insects may be fixed more rapidly by injecting the fixing solution directly into the body cavity with a hypodermic

Fig. 2. Photomicrograph of a portion of a celloidin section of the brain of the roach, *Periplaneta americana*, showing the extent of penetration of the stain when injected by the method described. Thickness—20 μ, Magnification—×500.

needle. This also distends the body sufficiently to prevent distortion caused by muscle contraction.

The specimens should be left in the fixing solution from 3 hours to over night depending on their size. They are then washed briefly in water and upgraded in alcohol to 70% by the usual method for histological material. As the dye is insoluble in alcohol, the stained material may be left in 70% alcohol indefinitely.

Sections may be made either by the celloidin or paraffin method. Safranin makes an excellent counterstain.

Examination of a celloidin section of the brain of the roach, *Periplaneta americana* (Fig. 2), shows the extent of penetration. The tracheal tubes may be traced, under oil immersion, to a point where they measure only 0.2 μ in diameter. This demonstrates that all the tracheae and many of the tracheoles are stained.

The use of this method is limited to those forms which have the holopneustic or hemipneustic type of tracheal system. For this reason many of the larval forms of *Diptera*, *Neroptera* and *Odonata* can not be injected by this method.

NOTES ON TECHNIC

A Simple Method for Mounting Embryological Material. During the course of some experiments conducted by the author for the past 2 years on the demonstration of centers of ossification in various mammalian embryos and fetuses, it became increasingly evident that a suitable method for mounting the specimens for permanent preservation was needed.

The well-known clearing methods utilizing KOH and glycerin were used, together with the respective staining methods for either bone or cartilage preparations. The final process of clearing and preserving was accomplished in pure glycerin (U. S. P.). Since the embryos varied greatly in size (in terms of crown-rump measurement) it was necessary to cut various sizes of glass plates to mount the finished specimens. This proved to be expensive and impractical, especially for material whose crown-rump measurements were less than 30 mm. Resort was finally made to the following procedure:

Sheet celluloid (as used in the automobile industry) was substituted for the glass slides or plates. The convenience of cutting the sheet celluloid to any desirable size and yet have a clarity equal to glass was soon realized. If, for example, an embryo whose crown-rump measurement was 45 mm. was to be mounted in glycerin in a museum jar, it was only necessary to cut the celluloid strip a bit larger and as wide as was needed. By tying the embryo to the celluloid support with the use of thread encircling the body, it was found to hold the specimen in a sturdy but natural position.

In a larger fetus, notches cut in convenient positions on opposite sides of the celluloid mounting strip afforded an adequate support for the specimens.

Buoyancy due to the density of the glycerin is eliminated after a day or so by penetration of the mounting medium into the specimen. —Clarence W. Nichols, Jr., Santa Cruz, California.

Old Gruebler Hematoxylin and Eosin Compared with Current American Stains.—It was the recent good fortune of the writer to be given two bottles of stains marketed by Dr. G. Gruebler & Co. of Leipsig and imported by a Pittsburgh physician sometime before 1908 when they were stored in his attic. As the parchment covers of the brown glass bottles had not been opened the stains appeared to be in perfect condition. One bottle contained 300 grams of "Haematoxylin pur. cryst." and the other an equal amount of "Eosin w. gelblich."

Special interest attaches to these samples because they were purchased during Dr. Gruebler's lifetime and not many years after he left the business. At that time "Dr. Gruebler's Laboratory" (which subsequently became K. Hollborn and Sons) and "Dr. G. Gruebler and Co.", altho existing as separate concerns, were in cooperation[1]. Because of the high repute of the Gruebler stains of those days, a comparison of these with current American stains appeared to be desirable.

The hematoxylin was examined in comparison with a sample of "Hematoxylin C.P." marketed by the Coleman and Bell Co. The Gruebler stain is coarsely granular, the particles varying in size from a powder to masses nearly a centimeter in diameter. In making the traditional .5% solution of hematoxylin the Gruebler stain dissolved more slowly in 95% alcohol, requiring 3 hours for complete solution to 1½ hours for the American stain. The straw color of the alcoholic solutions and of the diluted aqueous solutions was practically identical. On well fixed tissues, using the stains according to Heidenhain's iron hematoxylin method, a definite black staining of the chromatin and of chromosomes was obtained without obscuring coloration of the cytoplasm. The two stains also gave solutions of like color and staining quality when used as Delafield's hematoxylin solution. The results were so nearly identical as to indicate that the two stains are of equivalent staining quality.

The eosin stain is a fine powder similar in appearance to a sample of eosin Y, certification No. NE-4. of the National Aniline and Chemical Company. The Gruebler stain has about the same solubility in water and in 95% alcohol as the "water and alcohol soluble" American stain and the solutions are identical in appearance. Either in an aqueous solution or in an alcoholic solution the stains behaved similarly suggesting near equivalence in quality.

On the basis of these trials it appears that those who use certified American stains today have the equal in reliability of the famous Gruebler[2] stains of the first decade of the century.—T. M. McMillion, Geneva Col., Beaver Falls, Pa.

[1]The writer is indebted to Dr. H. J. Conn for supplying information about the history of the German firms.

[2]Samples of the Gruebler hematoxylin and eosin mentioned have been added to the collection of the Biological Stain Commission. Investigators who wish samples of the stains for comparative studies may obtain them from the writer on condition that the results of trials be reported to the Stain Commission.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

JOHANSEN, DONALD A. **Plant Microtechnique.** 6x9 in. 523 pp. Cloth. 110 illustrations. McGraw-Hill Book Co., Inc. New York, N. Y. 1940. $4.50.

This book, with about 500 pages of text, is perhaps the largest publication that has yet appeared in this country dealing exclusively with the field of botanical microtechnic. It is very attractively put up and illustrated largely from photographs of microscopic material prepared by the author. As he has spent much of his time in recent years preparing and marketing microscopic specimens, the material from which these photographs are prepared is excellent, and his experience with the microtechnical methods involved qualifies him to write on the subject.

The book is divided into two sections: the first of approximately 200 pages on "General Methods", and the second, of about 300 pages, entitled "Special Methods for the Various Phyla". The first of these two sections is a very adequate presentation of the methods employed in plant microtechnic with formulae of the most important solutions used in these methods; two of the sixteen chapters are devoted to stains and staining procedures. The second section of the book contains fifteen chapters, each dealing with special methods needed in the study of one of the large divisions of plants. Altho this is a somewhat unusual method of presenting the subject, it should prove useful to the student in this field.—*H. J. Conn.*

MICROSCOPE AND OTHER APPARATUS

BORRIES, B. v., and RUSKA, E. **Aufbau und Leistung des Siemens-Übermikroskopes.** *Zts. wiss. Mikr.*, **56**, 317. 1939.

This article presents a concise, informative description of the new Siemens' supermicroscope compared with his two earlier models built by the same firm. This instrument is about as compact as a modern X-ray machine and but slightly more difficult to operate. Two photographs of the apparatus with diagrams comparing the conventional and electron microscopes, together with the description of the construction and operation of the latter, are included.—*J. M. Thuringer.*

MASON, W. **Apparatus for cutting frozen sections on the rocking microtome.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 86–8. 1939.

An apparatus is described for freezing sections which does away with the necessity for a freezing microtome. The object-holder of the rocking microtome is modified to allow of the application of CO_2 by cutting away the jaws into which the wooden blocks are clamped. Upon the resulting flat top and directly over the hole in the center is soldered a metal cap about 3 cm. in diameter by 1.5 cm. deep with holes in the sides which allow CO_2 to escape. The object-holder is attached to the CO_2 cylinder by means of a brass rod 3.5 cm. long, having an external diameter such that it just fits the shaft of the object-holder. A hole 3 mm. in diameter is drilled thru the center of the rod, and the lower part is threaded to fit a brass nut having the same size thread as the CO_2 cylinder. If the gas is applied in a steady gentle stream, the interior of the cap fills with CO_2 snow and the tissue will remain frozen long enough for sectioning.—*Jean E. Conn.*

REESE, J. D. **A useful apparatus for staining slides.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 83–5. 1939.

An apparatus has been designed for staining slides which enables one to adjust the rack to a horizontal position and permits the dye to be poured back into the bottle without waste. A ring stand is used which has an upright bent at right angles about 2 in. from the end. A clamp holder is attached to the bent portion. The slide holder is made of stout brass wire, rectangular in shape, with a short metal rod attached to one end. By placing the rod in the free arm of the clamp holder, the rack may be easily adjusted to the horizontal. Small knobs of solder placed at intervals of about an inch along the rack keep the slides apart. A piece of monel metal with small metal "stops" opposite the spaces for the slides is soldered to one side of the rack in a slightly inclined position. When the rack is tilted, this piece of metal acts as a drain allowing the dye to flow down the incline into a bottle.—*Jean E. Conn.*

PHOTOMICROGRAPHY

KROGH-CHRISTOFFERSEN, A. **Das Mikrophotographieren ohne photographische Geräte.** *Zts. wiss. Mikr.*, **56**, 301. 1939.

Photomicrography, without the use of cameras, by projection of the microscopic image directly upon suitable projection paper is described. A contact print from the paper negative obtained produces the finished picture. Any one interested in this method is referred to articles on the well-known paper negative processes.—*J. M. Thuringer.*

SMITH, HILTON A. **A technique for making photomicrographic prints in color.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 45–51. 1939.

The author describes a method of adapting Eastman's "Wash-off Relief Process" to photomicrography. Since most sections are stained only in two colors, they can be reproduced by using only two negatives instead of three as is usually required. This method is described for tissues stained with hematoxylin and eosin, altho it may be adapted for other stains by using different filters. The A negative is exposed with the red light of two Wratten filters in combination, B (No. 58) plus E (No. 22). The corresponding positive is printed in the complementary color, a shade of blue, and reproduces the hematoxylin-stained nuclei. The red eosin is reproduced by the B negative which is exposed thru green filters. The writer found B (No. 58) plus H (No. 45) to be satisfactory; the complementary color, used in printing, is of eosin shade. The type of film preferred by the author is Process Panchromatic for the A negative and Panatomic for the B negative.

Chromatic aberration may be prevented by making a slight change in length of camera bellows between the two exposures. With a Bausch and Lomb type H camera, a 16 mm. aprochromatic objective and an 8× compensating eyepiece, the bellows will need to be about 1.5 mm. shorter for the B picture than for the A. The exact amount, however, must be determined by experiment.

In selecting dyes, Eastman's "A" dye (blue) was found satisfactory, but not the "B" dye. To replace the latter, the writer uses a mixture of orange G (C. I. No. 27), 2 vol., Bordeaux red (C. I. No. 88), 1 vol., and amaranth (C. I. No. 184), 1 vol. The total mixture is used in a 0.3% aq. solution.

For further details the author refers to publications of the Eastman Kodak Co. —*Jean E. Conn.*

DYES AND THEIR BIOLOGICAL USES

HÖBER, R. and BRISCOE-WOOLLEY, P. M. **Conditions determining the selective secretion of dyestuffs by the isolated frog kidney.** *J. Cellular & Comp. Physiol.*, **15**, 35. 1940.

The influence of the molecular configuration of a number of sulfonic acid dyes of the mono-azo, dis-azo and triphenylmethane groups on their secretion by the proximal tubules of the isolated Ringer-perfused frog kidney has been studied.

The sulfonate derivatives of mono-azo and dis-azo dyes are secreted by the kidney tubules and are found in the secretion in a concentration higher than that injected into the renal portal vein, provided that the sulfonate groups are arranged in a special manner. The triphenylmethane dyes do not undergo any secretory transport across the kidney tubules. Lipoid solubility of the dyes favored their secretion but was not indispensable.

From the data it appears that for active secretion of the dyes to occur, a bilateral elongated molecule, with the sulfonate groups on one half of the molecule, is necessary, whereas if sulfonate groups are located at the two ends of the molecule, the secretion of such dyestuffs is prevented. It is suggested that the first stage in the process of secretion is the anchoring of the dye on the cell surface by the organophilic non-polar portion of the molecule, the hydrophilic polar part (containing the sulfonate groups) attaching at the aq. surface.—*L. Farber.*

KROLL, H., STRAUSS, S. F., and NECHELES, H. **Concentration and detection of a dye in abscesses.** *Proc. Soc. Exp. Biol. & Med.*, **43**, 228–34. 1940.

To 100 mg. of an acid dis-azo dye, T 1824 (Evan's blue), in 15 cc. of water, aq. bromine was added, drop by drop, until the theoretical amount (2, 3 or 4 atoms) had been taken up. This was injected intravenously into dogs having abscesses, produced within 24 hr. after the injection of bacteria and foreign material. The abscess tissue was analyzed 20 hr. later. Best results were secured with monobrominated dye. Fixation of the dye by abscess tissue was regarded as due to absorption on the cell membrane and retention by solution of dye in the lipoid material of the cell wall.—*M. S. Marshall.*

LISON, L., and FAUTREZ, J. **L'étude physicochimique des colorants dans ses applications biologiques.—Etude critique.** *Protoplasma*, **33**, 116–51. 1939.

This is a critical analysis of the way in which many biologists have used dyes with little or no understanding of the varied properties of the dyes and have drawn conclusions which tend to be erroneous. An example given is the use of dyes in testing the lipoid nature of cell membranes by comparing the penetrability of the dyes with their solubility in fatty materials, without appreciating the variability of dye solubility in different fats and oils. There are also the investigations on the ultra filter theory by comparing intracellular penetration with the diffusibility of the dyes in solution without considering the nature of the diffusion medium. The authors stress the importance of understanding the physicochemical properties of the dyes, especially in biological media.

The length of the article limits a description to an enumeration of the chapters as follows: (1) Nomenclature and classification; (2) Chemical reactions and transformations, e.g., reducibility, formation of basic carbinols (pseudobases) and metachromasia; (3) Diffusibility and dispersibility in water, gels and dialysis; (4) Liposolubility, varying according to the lipoid material used and the possibility of hydrophily of some lipoids; (5) Electric charges; (6) Flocculability; (7) Electrocapillarity and surface activity (tensio-activité); (8) Physicochemical modifications in colloidal media, especially in serous media. A list of over 100 references is appended.—*Robert Chambers.*

MIRIMANOFF, A. **Remarques sur la secretion des tentacules de Drosera. Notes histochimiques.** *Protoplasma*, **33**, 211–14. 1939.

The author tested the reaction of the secretion to various agents by several methods, the best of which he found to be the following. Filter paper was moistened with a given reagent, then dried and a leaf of Drosera placed on the filter paper so that only the tips of the tentacles were in contact with the filter paper. Any reaction which took place at the points of contact was considered to be the result of the secretion at the tips of the tentacles. The reagents used were silver nitrate, parachlor-indophenol, o-nitroso-nitro benzol, ferric chloride, sodium nitro-prussate, Fehling's solution and potassium ferricyanide with ferric chloride. All showed the presence of a reducing substance. The results obtained convinced the author that the secretion contains ascorbic acid.—*Robert Chambers.*

ANIMAL MICROTECHNIC

COOKE, JEAN V., and BLATTNER, RUSSELL J. **Vital staining of virus lesions on chorio-allantoic membranes by trypan blue.** *Proc. Soc. Exp. Biol. & Med.*, **43**, 255–6. 1940.

One cc. of trypan blue, 0.5% aq., is placed on the membrane of the infected embryo; the egg is gently rotated and incubated 10–30 min. The membrane is removed, washed in saline and fixed flat in 10% formalin for a few minutes. The membrane is then drained, flattened on a 2x2 in. slide and mounted in glycerin gelatin, (50% glycerin, 5% gelatin, 1% phenol). Mounting is done at 70° C. The edges are sealed with balsam or asphalt.—*M. S. Marshall.*

INGLEBY, HELEN, and HOLLY, CLAIRE. **A method for the preparation of serial slices of the breast.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 93–6. 1939.

The following method is described for making serial sections from whole breasts: Fix breast by suspending it in a gauze sling in a jar of 4% formaldehyde; leave jar at least 24 hr. in the refrigerator room; remove breast from solution, wrap in a wet cloth and freeze. (The commerical ice cream freezer is handy for rapid freezing. The slicer put out by the American Slicing Machine Co. is the most satisfactory for making the serial slices.) After slicing, pile sections in order. (Difficult sections may be received in a bowl of water, floated onto numbered pieces of paper and stacked.) Fix sections a few hours; rinse in dist. water; stain 24 hr. in a single layer in a filtered solution of 5 cc. Harris' hematoxylin in 100 cc. dist. water; turn once to insure even staining; differentiate in acid alcohol; wash until blue; 70% alcohol, 1–2 hr.; 95% alcohol, 6–8 hr. (2 changes); abs. alcohol, 2–4 hr. (2 changes); benzine, 2–4 hr. (2 changes); store sections in mineral oil until required for use. Then transfer to oil of wintergreen (2 vol.) and benzol benzoate (1 vol.) for about 1 hr. before examining under the dissecting microscope. Masson's iron hematoxylin is an alternative stain recommended if photography is contemplated. After study sections must be returned to mineral oil.

The advantages of this method are: Every part of the tissue may be examined grossly; a three dimensional view is obtained; a whole breast can be ready for examination in 5 days; paraffin sections can be made at any time from any part if higher magnification is desired.—*Jean E. Conn.*

JALOWY, BOLESLAW, and CHRZANOWSKI, BRONISLAW. **Einige Bermerkungen über den Vorversilberungsprozess.** *Zts. wiss. Mikr.*, **56**, 334. 1939.

The authors review the theories advanced concerning the action of silver salts in impregnation processes (primary and secondary impregnations). Their work deals with the action during primary impregnation of tissues (preargentation as distinguished from postargentation when the silver salts are applied after preliminary treatment with suitable fixative or mordant). They conclude, on the basis of their investigation, that during preargentation the chlorine ions play the exclusive role in forming light sensitive AgCl.

They demonstrate that chlorine ions could be rapidly removed from the mesentery and other tissues by rinsing in large quantities of dist. water or by previous fixation with 10% or 20% formalin.

The AgCl formed during preargentation is converted into metallic silver under the action of light. This process does not begin spontaneously and when inaugurated thru light does not progress further. Pure silver albuminates do not darken with the action of light; their role is limited to the formation of a stroma (substrate) in which the granules of metallic silver are deposited. Silver phosphate, ortho-, meta-, and pyro-phosphate are likewise insensitive to light.—*J. M. Thuringer.*

KRAMER, FRANK M. **Macroscopic staining of anatomic and pathologic specimens.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 72–8. 1939.

Methods are described for the macroscopic staining of fat, iron, calcium, amyloid, and the brain. The following method is recommended for staining fat: Fix in formalin; cut a fresh surface and immerse in a sat. solution of Sudan III

or "scharlach R" (i.e. Sudan IV) in 70% alcohol until the fat has assumed a brilliant red color; avoid overstaining; decolorize in 95% alcohol for 12 hr. or more; wash thoroly in running water; mount in weak formalin. Fat-products of degeneration are also stained, but normal fat is stained more intensely.

For iron, the Berlin blue method is recommended as follows: Place specimen in a freshly prepared solution of equal parts 2% $K_4Fe(CN)_6$ and 1% HCl, until the desired reaction takes place; wash; preserve or mount in 70% alcohol.

The following silver nitrate method is recommended for staining calcium: Wash formalin-fixed specimen at least 24 hr. in running tap water; process thru several changes of dist. water for about 24 hr.; stain in the dark for 6–15 hr. in 1% $AgNO_3$ in dist. water; rinse in dist. water without exposing to light; place for a full day in 5% hypo solution; wash thoroly; mount in 50% alcohol or Kaiserling's preservative. Calcium may be stained a vivid pink color by alizarin dyes as follows: Stain 12 hr. in a 1:10,000 solution of alizarin red S, made basic by adding a small amount of KOH; differentiate for several days in equal parts of alcohol and glycerin, exposing jar to sunlight; mount in an alkaline preservative, e.g. Kaiserling's with a small amount of KOH added (1:1000).

Immersion in Lugol's iodine solution stains amyloid an intense brown, made sharper by adding weak H_2SO_4. To prevent fading, equal parts of 2% Lugol's and 1% H_2SO_4 are used as preservative.

The best method for staining the brain is as follows: To prevent tinting of the white matter, first process the specimen for a few min. in a phenol solution (phenol crystals, 80 g.; HCl, 3 cc.; $CuSO_4$, 10 g.; dist. water, 2000 cc.); immerse in cold tap water; stain in 0.5% nigrosin solution; rinse and examine occasionally to determine progress of staining; when the correct intensity is reached, remove "excess" dye with weak alcohol; rinse in running water; mount in weak formalin. This is the only method in which the intensity of staining can be favorably controlled.—*Jean E. Conn.*

LENDRUM, A. C., and McFARLANE, D. **A controllable modification of Mallory's trichromic staining method.** *J. Path. & Bact.*, 50, 381–4. 1940.

By applying the stains of the Mallory technic one at a time the effects desired may be attained with greater assurance. For the nuclear stain the following procedure is used: Allow 2.5 g. iron alum to dissolve over night at room temp. in 50 cc. dist. water. Add to this 0.25 g. celestin blue (C. I. No. 900) and boil the mixture 3 min. Filter when cool, and add 7 cc. glycerol. Stain in this solution 10–20 min. Rinse in water. Add filtered Mayer's hemalum; stain 5–10 min. Rinse in 95% ethanol. Differentiate in acid alcohol (1% HCl in 95% ethanol) until the red color ceases to come off.

After this step, the cytoplasm is stained as follows: Treat with 0.2% orange G in 80% ethanol saturated with picric acid, 2 min. to 16 hr. Rinse in water 30 sec. to 2 min. From a filter add fuchsin-ponceau solution (prepared by mixing 1% acid fuchsin in 1% acetic acid with 1% ponceau 2R, C. I. 79, in 1% acetic, and adding 2.5 cc. of 10% Na_2SO_4 to each 100 cc. of dye solution to inhibit mold growth). Stain for 15 sec. to several min. Rinse in 1% acetic acid and examine. Decolorize connective tissue in 1% phosphomolybdic acid; do not decolorize completely. Tissue fixed without chromate is sufficiently decolorized in 1–2 min. Stain in 2% soluble blue (C. I. 706) in 1% acetic acid, 2–10 min.; or in fast green F.C.F. in 1% acetic, 2–10 min. Rinse in 1% acetic acid and examine; if satisfactory, dehydrate rapidly and mount.

By this technic the nuclei stain dark red and the blood is colored distinctly different from the fibrin. This nuclear stain is more resistant to the action of picric acid than iron alum hematoxylin. The celestin blue solution should be replaced every 6 months; the other stain solutions last longer. The nuclear stain may appear to be entirely removed in the acid alcohol, but a mordanting effect is achieved in the nucleus that is not affected by picric acid and which fixes the acid fuchsin. Excess fuchsin staining may be remedied by immersion in the picric-orange-G solution. To emphasize color differences for photography, sections may be mordanted before staining in Hollande's solution without acetic acid. Poorly stained or faded sections can be re-stained by immersion in picric orange G or in a saturated solution of picric acid in 80–100% alcohol for 30–60 min., then rinsed and restained. Biebrich scarlet can replace the fuchsin-ponceau mixture. "Revector" dyes were used by the writer.—*S. H. Hutner.*

LILLIE, R. D. **The effect of hydrogen-ion concentration of formaldehyde used in storage for varying periods on staining of tissue.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 89–92. 1939.

The author reports experiments which show that formaldehyde solutions buffered to pH 7.5 preserve staining reactions of tissues for Romanowsky staining in particular and for nuclear staining in general much better than does formaldehyde not so buffered. The need for further work on this point is indicated.—*Jean E. Conn.*

MICHAEL, E. G. **Rapid method of staining frozen sections of tissues requiring immediate diagnosis.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 47–9. 1940.

The writer recommends the following rapid technic: Put tissue in 20 cc. of 10% formalin, boil 1 min. Cut sections and put in dist. water in a Petri dish. Put a section on a slide in dist. water in another Petri dish. Blot, cover with celloidin in alcohol-ether, and blot again. Put in dist. water, 20 sec. Remove. Cover with filtered Delafield's or Ehrlich's hematoxylin, 20 sec. Rinse in dist. water. Cover with 1% eosin Y in 95% alcohol, 15 sec. Add 3 drops of Mallincrodt's new beechwood creosote without removing the eosin. As soon as it penetrates, add 1 drop of Canada balsam in xylol and apply a cover slip. Wipe off excess mixture of eosin, creosote and balsam with a piece of gauze moistened with xylol.—*G. H. Chapman.*

ÖKLAND, FRIDTHOF. **Untersuchungen über Osteoblasten in Schliffen und Ausstrichen.** *Zts. wiss. Mikr.*, **56**, 345. 1939.

This work is based on the Rupprecht and Krompecher methods of preparing ground bone sections with combined fixation and staining. The author suggests the following modifications. Thin pieces of cranial vault of mouse, rat, or guinea pig (5 mm. sq.) are fixed for 24 hr. each in 60%, 95%, and abs. alcohol, and xylol. They are then ground on small carborundum hones saturated and kept wet with xylol. The thin sections are rinsed in clean xylol and brought into abs. alcohol, 5 min.; methyl-green-pyronin (Pappenheim, Grübler), 24 hr.; abs. alcohol, 10 min.; xylol, 1 hr.; neutral balsam. Results may be varied by previous *in toto* staining with Nile blue sulphate (sat. solutions in 60%, 95%, and abs. alcohol), then proceeding as above including the methyl-green-pyronin stain after grinding sections.

Bone marrow smears for control preparations may be stained with May-Gruenwald (similar to Wright's) or in the following way: a) methyl-green-pyronin, 3–5 min.; b) rinse in dist. water; c) abs. alcohol, xylol, and neutral balsam.

Results: cytoplasm stains red; characteristic vacuole, colorless.—*J. M. Thuringer.*

PARMENTER, C. L. **Chromosome numbers in Rana fusca parthenogenetically developed from eggs with known polar body and cleavage histories.** *J. Morph. and Physiol.*, **66**, 241–60. 1940.

Modifications of Bouin's solution (B-3 and B-15 without urea) are used in fixation. Since alcohols shrink the jelly into an unremovable, thin, tough coat, the eggs should be washed and preserved in 4% formalin, with frequent additions of Li_2CO_3 during washing. The following procedure is recommended: Before embedding, place eggs in 35% alcohol and dissect jelly from the eggs with very fine insect needles. Dehydrate and clear eggs in 30-min. successive baths of ⅓ dioxan, ⅔ dioxan, and 2 changes of pure dioxan. Infiltrate successively in equal parts of dioxan and soft paraffin at 47° C., soft paraffin at 47° C., and hard paraffin or tissuemat at 56° C. for about 15 min. each. Section eggs at 13 μ, with the knife passing thru both poles simultaneously. Of several stains used, Heidenhain's hematoxylin without counterstain, and safranin followed by light green or fast green give the most satisfactory results.—*Elbert C. Cole.*

PERRY, I. H., and LOCHHEAD, M. S. **Histological technique for the pituitary gland of the mouse.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 101–3. 1939.

A reliable differential stain for the pituitary of the mouse is described as follows: Remove the gland as rapidly as possible from the anesthetized animal by reflecting back the skin over the head and cutting the skull from its base. Cut out a wedge of bone around the pituitary and put into the fixing solution without handling. After hardening 15–30 min., dissect from the bone with needles under a binocular microscope without removing from the fixative. Transfer the pituitary with a pipette.

Fixation and embedding: Fix 4 hr. in Zenker-formaldehyde (95 cc. Zenker plus 5 cc. neutral 39% formaldehyde. Add the formaldehyde to the Zenker immediately before use). Wash 4 hr. in dist. water (many changes); dehydrate in 50% and 60% alcohol for ½ hr. each; leave over night in 70% alcohol; 70% iodized alcohol, 1 or 2 hr.; 80%, 90% and 100% alcohol, 2 hr. each; ether-alcohol, 2 or 3 hr. at 36° C.; one day each in 10%, 25% and 50% nitrocellulose, or 2%, 4%, 6%, 8% and 16% celloidin; harden, and cut at 4 μ.

The slides are stained using the technic described by Koneff (Stain Techn., **13**, 49–52, 1938) with the following modifications: Before staining, leave 12 hr. in 3% potassium bichromate, and rinse in dist. water. In step 1 treat with anilin alcohol 18 hr. instead of 45 min. In step 3 leave in azocarmine solution 4 hr. at 56° C. and 14 hr. at room temp.

The results are: Basophiles light blue with bluish-red nuclei; two types of acidophiles shown, one stained orange, the other deep red; nuclei of the acidophiles pink, bluish red or dark red; chromophobes gray with nuclei usually red; mitochondria in the basophiles always stained red.—*Jean E. Conn.*

SCHALM, O. W., and HARING, C. M. **A technique for reducing soft-tissue organs to thin serial slices, with special reference to its use on bovine mammary glands.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 97–100. 1939.

A technic is described by which the entire bovine udder may be reduced to serial slices. The procedure is as follows: Remove the entire mammary gland at slaughter; milk out as much secretion as possible; inject the duct and vascular systems with 8% formaldehyde, using a 5-liter aspirator bottle fitted with a rubber bulb for raising the pressure in the bottle. Inject the vascular system first thru the mammary artery of each mammary half; ligate the stumps of the large vessels as the formaldehyde escapes; continue injection until all the vessel stumps are so closed. Inject the duct system of each quarter thru the teat canal until the quarter is distended and tense. A round wooden applicator may be inserted into the teat canal to avoid distortion during hardening. Suspend the entire udder in 4% formaldehyde until thoroly hardened (2–6 weeks); separate the mammary halves along the median line; trisect the halves by cutting thru the center of each quarter from the tip of the teat to the base of the gland; slice each piece in an electrically-driven meat-slicer, making the slices from 3–5 mm. in thickness; attach paraffin-coated tags to each slice for identification; wrap together in cheesecloth; store in 4% formaldehyde. The procedure may be suspended at any stage as long as the tissues are kept submerged in paraffin.

The thin slices may be dehydrated in a graded series of either dioxan or tertiary butyl alcohol and infiltrated with paraffin. The slices are cooled between two glass plates, excess paraffin removed by gentle heat, and a protective coat of white shellac applied. Slices so prepared are best observed by transillumination.—*Jean E. Conn.*

VRAA-JENSEN, G. **Eine Method zur Doppelfärbung von Übersichtspräparaten des Zentralnervensystems.** *Zts. wiss. Mikr.*, **56**, 356–8. 1939.

This method is suited for "survey sections" of the central nervous system with excellent contrast between myelinated fibers and nerve cells. Sections are cut in paraffin (15 μ) and celloidin (20–25 μ).

Technic: 1) Deparaffinize. 2) Dist. water, 2 changes. 3) Stain in iron hematoxylin at 50–55° C. (paraffin sections, 10–15 min., celloidin sections, 20–30

min.). Prepare by mixing equal parts of 4% aq. $FeNH_4(SO_4)_2$ and 1% hematoxylin solution (10 ml. ripened solution hematoxylin to 90 ml. dist. water) immediately before use. 4) Tap water, 2 changes. 5) Differentiate in 4% aq. $FeNH_4(SO_4)_2$ (paraffin sections until the blue color is no longer visible thru the under surface of the slide; celloidin sections until the celloidin is colorless). 6) Tap water, 3 changes. 7) Differentiate, controlling with microscope, until cells just lose their stain in the following solution: 10 g. sodium borate, 12.5 g. $K_3Fe(CN)_6$ to one liter of dist. water. 8) Dist. water, 2 changes. 9) Rinse 5–10 min. in Vlesschouwer's pH 11 buffer solution (97.3 ml. of 0.53% aq. Na_2CO_3 and 2.7 ml. of 1.91% aq. $NaBO_3$). 10) Stain paraffin sections, 4 hr., or celloidin sections, 2 hr. in the following solution: Dissolve 0.1 g. Kernechrot (Hollborn), in 100 ml. 5% $Al_2(SO_4)_3$ by heating slowly, stirring constantly, and bringing to boil for 5 min.; allow to cool to room temp. and filter; add sufficient water thru the filter to bring the filtrate to 100 ml.; for every 5 vol. of the stain add 1 vol. *N*/1 NaOH. 11) Dist. water, 2 changes. 12) Dehydrate thru xylol and mount in balsam.

Results: Myelin sheaths, dark blue; nerve cells, connective tissue, non-myelinated fibers, and nuclei of neuroglia stain red; nerve cells stain most brilliantly while the intensity of the other structures gradually diminishes in order given. In the cerebrum the best results are obtained with celloidin.—*J. M. Thuringer.*

PLANT MICROTECHNIC

TANAKA, N. **Chromosome studies in Cyperaceae. VI. Pollen development and additional evidence for the compound chromosome in Scirpus lacustris L.** *Cytologia*, **10**, 348–362. 1940.

In connection with a cytological study of pollen development, the following recommendation is made:

After aceto-carmine stains, seal the cover-glass in place with melted "valap", a mixture of 2 parts vaseline, 2 parts lanolin, and 1 part paraffin, applied with a glass rod. If necessary, remove the valap with chloroform.—*Virgene Kavanagh.*

MICROÖRGANISMS

BRUNER, D. W., and EDWARDS, P. R. **Application of the endospore stain to blood smears from opsonophagocytic tests.** *J. Lab. & Clin. Med.*, **25**, 543–4. 1940.

This stain permits an accurate count of phagocytosed bacteria. A smear is prepared from the test material as follows: Spread the contents of a 2 mm. loop over 4 sq. mm. of a slide; air dry; fix by flaming 3 times; apply 5% aq. malachite green, 5 min.; wash 10–20 sec. in tap water; apply 0.5% aq. safranin, 10 sec.; wash quickly with tap water; dry and examine. The method is not applicable to all species of bacteria, for some do not retain the green in the presence of the safranin. Staphylococci are stained red. Streptococci are stained red for the most part but a few cells stain green. Some Gram-negative bacilli are stained red and others green. This technic gives excellent contrast between bacteria and white blood cells.—*John T. Myers.*

CANSEY, O. R. **Description of three species of frog microfilariae with notes on staining methods.** *Amer. J. Hyg.*, **30**, 117–21. 1939.

In the course of the writer's investigations of infected frogs, four stains for blood smears were employed: (1) Hemalum. Dried smears were dehemoglobinized in saline, placed in warm 70% alcohol for 15 min., passed thru tap water, then thru slightly alkalinized water and placed in the stain for 3–5 hr. They were destained in 70% alcohol containing 1% HCl until differentiated. After washing in tap water they were passed thru 70, 80, 95 and 100% alcohol, cleared 5 min. in xylol and mounted in balsam. (2) Methyl-green-pyronin: 0.2 g. methyl green and 0.075 g. pyronin in 100 cc. of isotonic NaCl solution. After being dehemoglobinized in saline, smears were placed in the stain for 12–36 hr., washed and mounted. (3) Azure-II-eosin. A stock solution of azure II was prepared

by adding 1 cc. each of 1% aq. azure II and 1% aq. Na_2CO_3 to 500 cc. of saline; this was diluted in 24 vol. of saline for staining. Dehemoglobinized smears were stained in the dilute azure for 3–5 hr., removed, and without washing, several drops of 0.025% eosin in saline added. A cover-slip was sealed in place with vaseline. (4) Vital azure II. Fresh blood was placed on a slide on which several drops of the stock azure II of No. 3 had been dried. After mixing, a cover-slip was put in place, and examination was made while fresh.—*John T. Myers.*

FITE, G. L. **The fuchsin-formaldehyde method of staining acid-fast bacilli in paraffin sections.** *J. Lab. & Clin. Med.*, **25**, 743–4. 1940.

The most reliable means of staining acid-fast bacilli in sections is by the following method, washing in tap water between each step: Place in a solution, containing new fuchsin 0.5 g., phenol crystals 5.0 g., alcohol (methyl or ethyl) 10 cc. and water to make 100 cc., for 12–24 hr. at 60° C. or 24–48 hr. at room temp.; transfer to freshly dist. aq. 5–30% formaldehyde, 5 min.; place in alcohol containing 2% of HCl, 10 min.; 1% aq. $KMnO_4$ until brown, usually 2–5 min.; 2% aq. oxalic acid, 1 min.; stain 2 min. in Harris' hematoxylin; then in Van Giesen's stain (acid fuchsin 0.1 g., picric acid 0.5 g., dist. water to make 100 cc.). Without washing, dehydrate in alcohol, clear in xylol and mount in balsam. Nuclei stain brown, connective tissue fibers red, muscle fibers yellow, and acid fast bacilli dark ultramarine blue. Lepra bacilli impossible to demonstrate by other methods were readily stained.—*John T. Myers.*

HAKANSSON, E. G. **A method of destroying the blastocysts (Blastocystis hominis) in fecal wet smears in order to facilitate the examination of Endamoeba histolytica.** *J. Lab. & Clin. Med.*, **25**, 546–7. 1940.

Blastocysts can be ruled out by making the fecal preparations in water instead of isotonic saline. Blastocysts and protozoon trophozoites will disintegrate but cysts will remain for several hours.—*John T. Myers.*

HORNUS, G. J. P. **Psittacose pulmonaire expérimentale de la souris blanche.** *Ann. Inst. Pasteur*, **64**, 97–116. 1940.

The following procedure is recommended for staining elementary bodies concentrated from emulsified infected lung: Smear suspension on a slide; dry; fix with methyl alcohol; when nearly dry, rinse thoroly in tap water; stain in 1% toluidine blue. Rinse; differentiate in 1% eosin-orange (neither formula nor source of dye stated); rinse, dry and examine. Five min. in toluidine blue suffice for recognition of the elementary bodies; longer staining gives more intense colors. The bodies are violet blue.

A modified Dominici technic is described for staining elementary bodies in sections. Fix in Bouin; cut paraffin sections and bring down to water; mordant ½ hr. in Lugol's solution; decolorize with $Na_2S_2O_3$; rinse in water. Stain ¼ hr. in a mixture of 1% erythrosin, and 1% orange G. Wash in running water; stain 5 min. in 1% toluidine blue. Differentiate in 0.2% acetic acid until the sections are rose-colored. Dehydrate and mount. Protoplasm of the cells is rose, with nucleus violet and elementary bodies clear blue.

An alternative Giemsa method is as follows: Prepare sections after Bouin fixation; rinse in buffer (2% Na_2HPO_4, 1% KH_2PO_4). Stain in Giemsa solution freshly prepared by adding 25 drops of dye to 25 ml. neutral water. Stain 12 hr., then an additional 36 hr. in fresh stain solution. Wash in running water; differentiate in a mixture of 1% orange G and 5% tannic acid until the section has an orange tint. Change the latter solution once or twice as it becomes saturated with the Giemsa stain. The results are cytoplasm yellow, nuclei brown, and elementary bodies intense violet. A color plate illustrates these effects.—*S. H. Hutner.*

JOHNS, C. K., and HOWSON, R. K. **Potentiometric studies with resazurin and methylene blue in milk.** *J. Dairy Science*, **23**, 295–302. 1940.

Coefficients of correlation of the reduction test times and the Breed counts of individual cells on 369 samples of market milk were $r = -0.711 \pm 0.017$ for the

resazurin test, using a definite pink color as the end-point, and $r = -0.651 \pm 0.020$ for the methylene blue test. The tubes were inverted every two hours in the reduction tests. The ratio of the resazurin reduction times to the methylene blue reduction times was approximately 3:4. The shapes of the time-potential curves obtained with the two dyes indicate that the shorter reduction time with resazurin is due to the more rapid initial drop in potential which occurs. Shortly below the point where the change to pink takes place, the curve flattens somewhat before again declining to reach the same final potential level as the milk to which methylene blue was added. In the range immediately following reduction to the pink, resazurin seems to have a greater poising action than does methylene blue at the same level of potential. More rapid reduction as a result of inversion of the tubes at hourly intervals is due to more active growth of bacteria. The resazurin test apparently is more satisfactory than the methylene blue test for grading samples containing considerable numbers of slow-reducing thermoduric organisms.—*F. E. Nelson.*

MATUSZEWSKI, T., and SUPINSKA, J. **Studies on the methylene blue reduction test. II. Comparison between the old and the modified methods.** *J. Dairy Research*, **11**, 43–50. 1940.

Methylene blue reduction tests were made on each of 185 milk samples, using both the old method and the new method of Wilson, in which the tubes are inverted every half hour until reduction occurs. The new technic gave a shorter reduction time than the old, and smaller variations in the numbers of bacteria (determined by microscopic examination of stained preparations) corresponding to a given reduction time. The numbers of organisms actually present in the milks at reduction time approximated very closely the numbers calculated by means of equations whose derivation is presented. More bacteria were present at the time of reduction in the inverted tubes. Abnormally short reduction times, possibly due to aeration before the test began, and unusually long reduction times, apparently due to slowly active bacteria, were encountered at times. The authors believe the proposed modification constitutes an advance in the reduction test technic.—*F. E. Nelson.*

PARSONS, R. J. **The staining of Negri bodies in formaldehyde and alcohol fixed tissues.** *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, **19**, 104–8. 1939.

The following procedure is described as a simple and reliable stain for Negri bodies in tissue fixed in formaldehyde or alcohol: Fix in 4% formaldehyde 18 hr. to 7 weeks or in 96% alcohol 18 hr. to 3 weeks. (It is preferable to dissect out the hippocampal gyrus before fixing and to prepare several cross-sectional blocks. The cerebellum should always be fixed.) Float paraffin sections cut at 4–5 μ directly onto clean cover slips or slides; dry over night in a warm oven or warm over a flame until the paraffin just begins to melt; remove paraffin from sections and bring to 96% alcohol. Stain 2 min. in acidulated ethyl eosin prepared as follows: Add 1.25 cc. of a solution of 1.0 g. ethyl eosin (National Aniline & Chemical Co., certified) dissolved in 100 cc. ethyl alcohol 96% and filtered before use to 22 cc. of a solution of 0.6 cc. acetic acid (Reagent Special, 99.5% acetic acid) in 99.4 cc. dist. water. Wash off excess stain in 96% ethyl alcohol, leaving sections a medium pink color. Stain 2 min. in borax-methylene-blue (methylene blue, 1.0 g.; borax, 1.0 g.; dist. water, 100 cc.). Rinse briefly in dist. water. Differentiate 2–5 min. in 0.25% acetic acid (99.5% acetic acid, 1.0 cc.; dist. water, 400.0 cc.), making sections a pale bluish-pink. Rinse in 96% alcohol; dehydrate as rapidly as possible in 2–3 changes of abs. alcohol; xylol (2 changes); mount in balsam.

Results: Negri bodies bright orange-red with a vacuolated inner structure or a small blue central dot; Lyssa bodies bright orange-red, but uniformly stained; nucleoli dark blue; red blood cells an intense copper red; nuclei blue; Nissl substance bright blue; cytoplasm of nerve cells pale pink; neuroglia pale pink. The differential coloration of the Negri bodies is so striking that they can be found under low ($\times 100$) magnification, using higher magnification only for positive confirmation. This stain has been employed successfully on material from dogs and man.—*Jean E. Conn.*

STEINER, GABRIEL. A simple method of staining the spirochaetes in routine paraffin sections. *J. Lab. & Clin. Med.*, **25**, 204–10. 1939.

The following technic is proposed: Dehydrate tissue blocks in graded alcohols, clear in xylol, embed in paraffin, cut 9–10 μ thick and place in abs. alcohol (2 changes). Place sections in the following solution for 1–1½ min.: uranium nitrate (4% in abs. alcohol), 20 cc.; gum mastic (25% in abs. alcohol), 40–50 cc.; abs. alcohol, 20–30 cc. Wash in 3 changes of dist. water. Place in 0.1% aq. $AgNO_3$ for 1.0–1½ hr. at 100° C. Dehydrate in graded alcohols. Place in 10–12.5% gum mastic (in abs. alcohol) for 5 min. Wash 3 times in dist. water. Reduce for 5 min. in 5% hydroquinone (hydroquinone, 10 g.; 12.5% alcoholic gum mastic, 1 cc.; dist. water, 200 cc.) Counterstain, if desired, with hematoxylin and eosin, cresyl violet, or fuchsin. Dehydrate, clear and mount. The method is simple, rapid and free from precipitates.—*John T. Myers.*

TAYLOR, DEAN M. A study of procedures for detection of coliform organisms in Minnesota drinking water. *J. Amer. Water Works Assoc.*, **32**, 98–104. 1940.

In order to find a method of detecting coliform organisms in water, less laborious and time consuming than the *Standard Methods* "completed test", 1,534 samples of drinking water were tested using (1) that method, (2) brilliant green lactose bile and (3) fuchsin lactose broth. The second compared very favorably with the first and might advantageously replace it. For isolating coliform organisms, the alternative "completed test" of *Standard Methods*, employing brilliant green lactose bile, was preferable to the usual "completed test".—*Merritt N. Pope.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the April number of this Journal.

STAINS CERTIFIED MAR. 1, TO MAY 31, 1940*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Nigrosin WS	CNi–3	....	For negative staining of bacteria	Mar. 18, 1940
Chlorazol black E	NBc–1	....	As histological and cytological stain	Mar. 18, 1940
Eosin Y	CE–10	92%	As histological stain and as a constituent of blood stains	Apr. 1, 1940
Crystal violet	CC–10	90%	As histological, cytological, bacteriological stain, and in bacteriological media	Apr. 1, 1940
Carmine	LCa–2	....	As histological and cytological stain	Apr. 4, 1940
Carmine	NCa–7	....	As histological and cytological stain	Apr. 4, 1940
Alizarin red S	LAr–2	....	As stain for bone	Apr. 10, 1940
Congo red	NQ–8	87%	As histological counterstain	May 10, 1940
Nigrosin	NNi–7	....	For negative staining of bacteria	May 16, 1940
Eosin Y	NE–15	87%	As histological stain and as constituent of blood stains	May 23, 1940
Crystal violet	NC–21	90%	As histological, cytological, bacteriological stain, and in bacteriological media	May 24, 1940
Brilliant green	NBg–9	94%	For use in bacteriological media	May 31, 1940

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.